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Differential sensitivity of Italian ryegrass (*Lolium multiflorum*) cultivars to fenoxaprop

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Several seed production fields of the Italian ryegrass cultivar 'Tetrone' were destroyed in 1988 by 280 to 350 g ai ha^{-1} racemic fenoxaprop applied for wild oat control. Because similar rates of fenoxaprop had possessed adequate safety when applied to 'Oregon common' Italian ryegrass, suspicion arose that the cultivars differed in tolerance. Seedlings of 21 commonly grown cultivars were screened in the greenhouse at the three-leaf growth stage to determine their fresh weight GR_{50} for fenoxaprop. The GR_{50} values for the two most tolerant cultivars, 'Marshall' and 'Torero', were more than threefold greater than the two most sensitive cultivars, 'Futaharu' and 'Ace'. Cultivars could be separated into sensitive, intermediate, and tolerant groups, but the distribution of the GR_{50} values appeared to be continuous rather than discrete. Tolerance increased with growth stage, and the average GR_{50} for tillered plants was 80% higher than that for the two-leaf stage and 41% higher than that for the four-leaf stage seedlings. Cultivars differed slightly in the specific activity of acetyl–coenzyme A carboxylase (ACCase) (EC 6.4.1.2) and in the I_{50} values for the inhibition by fenoxaprop, but the only clear relationship between these biochemical factors and whole-plant tolerance was a threefold increase in ACCase activity at the tillered stage over that present in the younger seedlings.

Nomenclature: Fenoxaprop; wild oat, *Avena fatua* L. AVEFA; Italian ryegrass, *Lolium multiflorum* Lam. LOLMU.

Key words: Acetyl-CoA carboxylase, ACCase, growth reduction, GR_{50}, enzyme inhibition, I_{50}, R:S ratio.

Aryloxyphenoxypropanoic acid (APP) and cyclohexanedione (CHD) herbicides are important tools for the control of grassy weeds in dicotyledonous crops and also have utility in cereals and grass seed crops (Andersen 1976; Brewster et al. 1977; Mueller-Warrant 1990, 1991; Palmer and Read 1991). Both groups of compounds are potent inhibitors of the enzyme acetyl–coenzyme A (CoA) carboxylase (ACCase) (Gronwald 1991; Rendina and Felts 1988; Rendina et al. 1989), a biotin-containing high molecular weight (wt) multifunctional protein catalyzing the adenosine triphosphate (ATP)-dependent carboxylation of acetyl-CoA to malonyl-CoA in various pathways, including fatty acid synthesis (Harwood 1989; Stahl and Sparace 1991). The APP herbicide fenoxaprop was first registered in 1987 for selective control of wild oat, roughstalk bluegrass (*Poa trivialis* L.) and warm-season annual grasses in perennial ryegrass (*Lolium perenne* L.), Italian ryegrass, tall fescue (*Festuca arundinacea* Schreb.), and red fescue (*F. rubra* L.) grown for seed. With the exception of red fescue, all these crops experience temporary stunting and some degree of chlorosis when treated with fenoxaprop, and safety margins seldom exceed two to four times the label use rates. Tolerance to ACCase inhibitors in red fescue is based on the differences between it and the other grasses in herbicide binding to the enzyme, and the resistant to susceptible ratios (R:S) between red fescue and the other grasses are similar to those seen between resistant broadleaved species and susceptible grasses, i.e., approximately 1000-fold (Butler and Appleby 1986; Stoltenberg et al. 1989). In the spring of 1988, several seed production fields of the Italian ryegrass cultivar 'Tetrone' in Oregon were destroyed by 280 to 350 g ha^{-1} racemic fenoxaprop applied for wild oat control. Because fenoxaprop had possessed adequate safety when applied at similar rates to the 'Oregon common' Italian ryegrass, suspicion arose that cultivars differed in their inherent tolerance to this herbicide.

Several mechanisms for resistance to ACCase inhibitors have been recognized in weedy *Lolium* spp. Altered ACCase has conferred high levels of resistance in Italian ryegrass (Betts et al. 1992; De Prado et al. 2000; Evenson et al. 1994, 1997; Gronwald et al. 1992). Enhanced diclofop metabolism was partially responsible for moderate levels of resistance in rigid ryegrass (*L. rigidum* Gaudin) (Menéndez et al. 1996; Powles et al. 1990; Preston et al. 1996; Shimabukuro and Hoffer 1991), but the ability to recover from herbicide-induced membrane depolarization was also critical (De Prado et al. 1999; Shimabukuro 1990; Shimabukuro et al. 1979). There is also evidence that tolerance to APP and CHD herbicides can be induced by the overproduction of the target enzyme (Parker et al. 1990; Shah et al. 1986). However, the activity of the extractable ACCase in a diclofop-resistant rigid ryegrass was not changed by the exposure of plants to the herbicide (Matthews et al. 1990). A wide range of R:S ratios for tolerance to diclofop in poison ryegrass (*L. temulentum* L.) and perennial ryegrass accessions from Arkansas was recently reported (Kuk et al. 2000). The most resistant type possessed an altered ACCase and an unknown mechanism conferring cross-resistance to chlorsulfuron, whereas the two least resistant accessions were only 1.3 and 2.4 times as tolerant as the susceptible check.

The manufacturer recently stopped the production of the mixed isomer, emulsifiable concentrate formulation of fen-

TABLE 1. Response of 21 Italian ryegrass (*Lolium multiflorum* Lam.) cultivars to fenoxaprop applied at the three-leaf growth stage.[a]

Cultivar or cultivar tolerance group (*n*)[b]	Mean relative fresh wt[c]	GR_{50}	R^2	Regression MSE	*F* test[d]	Relative tolerance[e]
	% of check	g ha^{-1}				
Futaharu	18 hi	63	0.85	167	44**	0.5
Ace	13 i	64	0.83	279	25**	0.5
Hitachioba	30 fgh	71	0.79	177	10*	0.5
Waseyutaka	17 hi	73	0.86	187	40**	0.6
Minamiwase	26 gh	78	0.95	58	178**	0.6
Tetrone	29 fgh	84	0.86	139	20**	0.6
Billiken	33 fg	90	0.86	136	16**	0.7
Yamaaoba	34 efg	105	0.70	352	19**	0.8
Lemtal RVP	42 cdef	127	0.66	368	8*	1.0
Sakurawase	40 def	128	0.59	385	6**	1.0
Barmultra	46 bcde	131	0.84	133	50**	1.0
Promenade	39 defg	136	0.71	381	18**	1.0
Bartolini	42 cdef	149	0.75	305	25**	1.1
Florida RR	50 abcd	151	0.53	368	6*	1.1
Barspectra	51 abcd	163	0.63	242	9*	1.2
Gulf	47 bcde	166	0.50	470	9*	1.3
Aubade	53 abc	176	0.70	203	16**	1.3
Florida 80	57 ab	182	0.81	121	26**	1.4
Ellire	55 ab	183	0.54	309	9*	1.4
Marshall	57 ab	219	0.54	382	10*	1.7
Torero	60 a	228	0.80	69	20**	1.7
Averages within groups (*n*)						
Sensitive (8)	25 c	79	—	—	—	0.6
Intermediate (5)	42 b	134	—	—	—	1.0
Tolerant (8)	54 a	184	—	—	—	1.4
Regression of data pooled within groups (*n*)						
Sensitive (8)	—	79	0.80	192	134**	0.6
Intermediate (5)	—	130	0.71	268	90**	1.0
Tolerant (8)	—	180	0.58	244	87**	1.4
Regression of all data adjusted as fenoxaprop rate divided by relative cultivar tolerance (*n*)						
All cultivars (21)	—	130	0.75	214	347**	1.0

[a] Abbreviations: MSE, mean square error; wt, weight.

[b] Sensitive group includes Futaharu, Ace, Hitachioba, Waseyutaka, Minamiwase, Tetrone, Billiken, and Yamaaoba; intermediate group includes Lemtal RVP, Sakurawase, Barmultra, Promenade, and Bartolini; tolerant group includes Florida RR, Barspectra, Gulf, Aubade, Florida 80, Ellire, Marshall, and Torero. Number of cultivars within each group is denoted by *n*.

[c] Mean relative fresh wt is the average of the data from all rates of fenoxaprop that averaged 168 g ha^{-1}. Mean followed by the same letter do not differ at the P = 0.05 probability level.

[d] Regressions based on pooled data from five to seven rates of fenoxaprop in two experiments. The symbols * and ** denote regression *F* test significance at the P = 0.05 and 0.01 levels, respectively.

[e] Relative tolerance is calculated as GR_{50} of the cultivar or group of cultivars divided by mean GR_{50}.

oxaprop registered for use in grass seed crops, and indicated that only an active isomer formulation containing a chemical safener would be supported for future registrations on any crop. Tests of several formulations of fenoxaprop enriched in the active isomer content showed enhanced activity on cool-season grasses and raised serious questions of crop safety in perennial ryegrass and tall fescue grown for seed (Mueller-Warrant 1991). Potential variation in the tolerance to fenoxaprop among Italian ryegrass cultivars will clearly pose additional complications in registering the new formulations. In light of these recent developments, we completed the analysis of experiments conducted from 1988 through 1990 to fully document the variation in varietal response to fenoxaprop.

Our first objective was to quantify the variation in tolerance to fenoxaprop among commonly grown Italian ryegrass cultivars. Our second objective was to characterize the effect of the growth stage on the tolerance of the selected Italian ryegrass cultivars to fenoxaprop. Our final objective was to examine the possibility that variation in tolerance to fenoxaprop at the whole-plant level was because of differences in the specific ACCase activity or the differential inhibition of ACCase by fenoxaprop.

Materials and Methods

Cultivar Tolerance to Fenoxaprop

Response of 21 cultivars (Table 1) to different rates of fenoxaprop was evaluated in the greenhouse in the late spring of 1988, and the test was repeated in the early spring of 1989. Seeds were obtained from the Oregon State University Seed Testing Laboratory, Corvallis, OR. Ten seeds were planted in each 10- by 10-cm plastic pot filled with a peat–loam–sand–pumice potting mixture (1:1:1:3 ratio by vol), with the pH corrected to 6.5. The pots, randomized

on greenhouse benches, were watered three times a day until germination and then once a day until harvest. Pots were gradually thinned after emergence to a final density of seven seedlings per pot. A soluble fertilizer was applied twice during the growth period. Temperatures of approximately 16 and 10 C during day and night, respectively, were maintained throughout the growing period. Natural light was supplemented with artificial light at 400 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) to maintain a minimum 12-h photoperiod.

Plants were treated with an emulsifiable concentrate formulation of racemic fenoxaprop at the three- to four-leaf stage in 1988 and at the two- to three-leaf stage in 1989 using a pressurized-air sprayer delivering 480 L ha^{-1}. Differences in seed germination rates and seedling growth rates resulted in a range of growth stages when treatments were applied, with most seedlings being in the three-leaf stage in both years. One untreated check was harvested at the time of fenoxaprop application for recording the initial wt, whereas the other two checks were harvested along with all the other treatments 4 wk later. Plants were harvested at a 1-cm cutting height to measure both fresh and dry wts. Visual phytotoxicity ratings were made shortly before the harvest. Relative fresh wt gain for each treatment was calculated, as described by Morrison and Maurice (1984), as follows, with Fwt_0 equal to fresh wt per pot at the time of spraying, Fwt_t equal to fresh wt per pot of the treatment at final harvest, and Fwt_c equal to fresh wt per pot of the check (mean of two checks) at final harvest:

$$\text{Relative fresh wt} = 100(Fwt_t - Fwt_0)/(Fwt_c - Fwt_0) \quad [1]$$

The experiments were laid out in a completely randomized design with a factorial arrangement of fenoxaprop rates (0, 56, 112, 168, 224, and 280 g ha^{-1}) and Italian ryegrass cultivars. On the basis of the tolerance to fenoxaprop in the first test, cultivars were divided into susceptible, intermediate, and tolerant groups for the repeat test. A higher rate range (84 to 336 g ha^{-1}) was applied to the more tolerant cultivars and a lower range (28 to 168 g ha^{-1}) to the more susceptible ones to better characterize the rate response curves of all cultivars. Treatments were replicated five times in both tests, except for the untreated checks, which were replicated 10 times. To improve precision in measuring cultivar tolerance to fenoxaprop, data from both tests were pooled after testing for homogeneity of variances. Relative fresh wt pooled over common fenoxaprop rates were subjected to analysis of variance, and means were separated using LSD at the 5% probability level. Treatment means from both tests were used in a pooled nonlinear regression of relative fresh wt vs. fenoxaprop rate to determine GR_{50} values for each cultivar. The regression model was

$$Y = C_{\text{asymptote}} + AB^X \quad [2]$$

In Equation 2, Y is the relative fresh wt, X is the fenoxaprop rate, $C_{\text{asymptote}}$ is the asymptote approached from above, and coefficients A and B are derived from regression of $\log(Y - C_{\text{asymptote}})$ vs. X. $C_{\text{asymptote}}$ was optimized by changing its value in successive regressions until the mean square error (MSE) was minimized. Both MSE values and coefficients of determination (R^2) were calculated on the untransformed data by summing the squares of the differences between the observed and expected values rather than on the log-transformed values.

Growth Stage Effects

The effect of the growth stage on tolerance to fenoxaprop was studied in subsequent tests using subsets of the original 21 cultivars. The two- and four-leaf growth stages were compared for 10 cultivars selected to cover the full range of tolerance found in the first studies. 'Futaharu', 'Ace', 'Waseyutaka', and Tetrone were chosen to represent the most sensitive group, 'Sakurawase' and 'Barmultra' were chosen to represent the intermediate group, and 'Gulf', 'Aubade', 'Marshall', and 'Torero' were chosen to represent the most tolerant group. Experimental techniques were similar to those used in the original studies, except for the addition of the growth stage factor. No split applications were tested. Growth stage experiments were first conducted in the winter of 1990, were repeated in the early summer of 1990, and were repeated again in a modified format in the late summer of 1990. Only five cultivars were used in the final test, but planting dates were staggered to obtain two-leaf, four-leaf, and tillered growth stages when fenoxaprop was applied. Results from an earlier test of treatments applied at the tillered growth stage were similar to those from the final test but were omitted because the application dates had been staggered after a common planting date to obtain the range of growth stages, and results were less uniform. Treatments were replicated three times in the growth stage studies except for the further duplication of the untreated checks. To improve the precision in measuring cultivar by growth stage tolerance to fenoxaprop, data from the first two growth stage tests were pooled after testing for homogeneity of variances. Greenhouse environmental control settings during the first growth stage test were similar to those used during the original cultivar tests. No supplemental lighting was supplied during the second and third growth stage tests, and a 20 C daytime temperature was maintained during cloudy weather but was frequently exceeded during sunny weather. Day length exceeded the 12-h minimum set by artificial lighting in all tests, except the first growth stage study, and daily PAR varied naturally with season.

Acetyl–Coenzyme A Carboxylase

Because of physical and financial limitations, 11 out of 21 varieties were chosen for laboratory analysis of ACCase. Varieties were chosen to span the full range of response to fenoxaprop present in the whole-plant studies. Plant material for enzyme assays was grown during the summer of 1991 and was extracted at the two-leaf, four-leaf, and tillered growth stages. Extractions were conducted on two sets of plants, with staggered planting dates and a common extraction date for one set and a common planting date with staggered extraction dates for the second set. At the two-leaf stage whole plants were harvested, whereas at the later growth stages only the two youngest leaves were used. The tissue was collected in the greenhouse, transported on ice, thoroughly washed with distilled water, wiped dry, and then ground in liquid nitrogen using a mortar and pestle. When the tissue was in a powdered form, a buffer was added in a wt/v ratio of 1:2.5 (fresh wt to buffer). The extraction buffer comprised 100 mM Tricine (pH 8.0, HCl), 15% (v) ethylene glycol, and 0.2% (v) 2-β-mercaptoethanol. The macerate was filtered through a single layer of Miracloth™. The filtrate was centrifuged at 14,000 × g for 30 min. The pellet

was discarded, and the supernatant was either used immediately or stored at − 20 C until use. Protein content (mg ml^{-1}) of the enzyme supernatant was assayed using the Bio-Rad method with bovine serum albumin as the standard (Bradford 1976).

ACCase activity was assayed as described by Stoltenberg et al. (1989), with minor modifications. The activity was assayed in reaction volumes of 250 μl in a fume hood by the acetyl-CoA–dependent incorporation of $NaH[^{14}C]O_3$[1] in 7-ml minivials in the presence of 0, 0.316, 1, and 3.16 mM fenoxaprop parent-acid (racemic mixture of *R* and *S* enantiomers). The reaction mixtures (final vol) contained 100 mM Tricine (pH 8.0, HCl), 0.5 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM ATP, 50 mM KCl, 3 mM acetyl-CoA, 15 mM $NaH^{14}CO_3$ (0.375 MBq μmol^{-1}), and 0.1 ml of the crude enzyme extract. The reaction was started with the addition of the enzyme and incubated at 35 ± 2 C for 15 min (Rendina and Felts 1988; Secor and Cséke 1988; Stahl and Sparace 1991). The reaction was terminated by the addition of 25 μl 12-M HCl. All steps of the enzyme assay, from the addition of the enzyme onward, were carried out in a fume hood. The reaction mixtures were subsequently dried in an evaporation rack to allow the vaporization of unreacted $^{14}CO_2$. After evaporation, the solids were redissolved in 2 ml boiling double-distilled water. Radioactivity incorporated into the acid- and heat-stable fraction was estimated by liquid scintillation spectroscopy after adding 5 ml of the scintillation cocktail[2] to this solution. The readings from the scintillation counter were corrected for background, counting efficiency, and acetyl-CoA– and ATP-independent incorporation of radioactivity. The I_{50} values (the fenoxaprop dose inhibiting the ACCase activity by 50%) were computed by regression analysis.

Results and Discussion

Cultivar Tolerance to Fenoxaprop

Symptoms of phytotoxicity occurred on all 21 cultivars when treated with sufficiently high rates of fenoxaprop. Symptoms included stem and leaf necrosis, chlorosis of younger leaves, and darkening of older leaves. These symptoms are typical of herbicides that inhibit fatty acid biosynthesis including fenoxaprop (Bhowmik 1986; Köcher et al. 1982; Schumacher et al. 1982). In untreated checks, the most vigorous cultivar Sakurawase yielded more than twice as much biomass as did the least vigorous cultivar 'Bartolini' (data not shown). Therefore, we expressed the yield for each cultivar as the percent of its own untreated check. Because visual phytotoxicity ratings, percentage reduction in dry wt gain, and percentage reduction in fresh wt gain were well correlated, only the relative fresh wt gain data are shown. Moderate but statistically significant variation in tolerance existed among the cultivars, with Torero yielding 4.8 times as much as Ace at common rates of fenoxaprop (Table 1). There was a significant interaction between the cultivar and the fenoxaprop rate for fresh wt gain when all 21 cultivars were analyzed together. This interaction occurred because many of the medium to high rates killed all seedlings of the most sensitive cultivars, whereas many of the low to medium rates failed to even stunt the growth of the most tolerant cultivars. This interaction disappeared when cultivars were separated into groups of the eight most sensitive, five intermediate, and eight most tolerant cultivars, and relative fresh wt was reanalyzed within these groupings. When treated with the rates of fenoxaprop averaging 168 g ha^{-1}, the average relative fresh wt of the eight most tolerant cultivars was 114% higher than that of the eight most sensitive cultivars, and 28% higher than that of the five intermediate cultivars.

Despite the interaction between the fenoxaprop rate and the cultivar, the mean relative fresh wt was still a more useful way to separate cultivars than simply using the raw means at each individual rate of fenoxaprop. The two problems with separating cultivars using data for each rate of fenoxaprop were that such separations were (1) not fully consistent across rates because of random error, and (2) less precise than the means. Torero, Marshall, 'Ellire', and 'Florida 80' were the most tolerant cultivars, with their mean relative fresh wt exceeding those of all eight cultivars in the sensitive group and those of four out of five cultivars in the intermediate group (Table 1).The relative fresh wt of Torero exceeded that of 14 of the 20 other cultivars. Futaharu and Ace were the most sensitive cultivars, with significantly lower relative fresh wt than two other cultivars in the sensitive group and all 13 cultivars in the intermediate and tolerant groups.

Regression analyses provided a clearer picture of the differences between cultivars in their response to fenoxaprop than did the mean relative fresh wt. GR_{50} values from the regressions ranged from 63 and 64 g ha^{-1} for Futaharu and Ace to 219 and 228 g ha^{-1} for Marshall and Torero, respectively. The public cultivar Marshall is a certified version of Oregon common Italian ryegrass, and finding it to be the second most tolerant cultivar explains why fenoxaprop appeared to be safe in early field tests conducted on Oregon common Italian ryegrass. Over 90% of Italian ryegrass acreage in Oregon is of the common variety (W. C. Young, III, personal communication). Similarly, the presence of Tetrone in the sensitive group is consistent with the injury reported in 1988. The GR_{50} value for Marshall was 2.6 times that for Tetrone. In addition to the Oregon common type, three Italian ryegrass cultivars were specifically listed on the fenoxaprop label as possessing adequate tolerance to treatment: 'Promenade', 'Barspectra', and Gulf. The GR_{50} values for all three cultivars exceeded the mean GR_{50} value of all 21 cultivars, although Promenade did fall in the intermediate group. The GR_{50} values for the sensitive, intermediate, and tolerant cultivars averaged 79, 134, and 184 g ha^{-1}, respectively.

Relative cultivar tolerance to fenoxaprop was calculated as the cultivar GR_{50} divided by the average GR_{50} for all 21 cultivars. Using this relative tolerance, it was possible to adjust the rates applied to each cultivar to a common scale by dividing the actual rate applied by the cultivar's relative tolerance. The GR_{50} value from regression of the adjusted data pooled over all cultivars was 130 g ha^{-1}, nearly the same as the overall mean from the 21 separate regressions, 132 g ha^{-1} (Figure 1; Table 1). *F* tests comparing the full model (separate regressions for each cultivar) vs. the reduced model (a single regression of the pooled, adjusted data) were nonsignificant, indicating that it was appropriate to summarize cultivar response to fenoxaprop with a graph of relative fresh wt vs. adjusted rate (Figure 1) and a list of relative tolerances (Table 1). The continuous distribution of the GR_{50} values

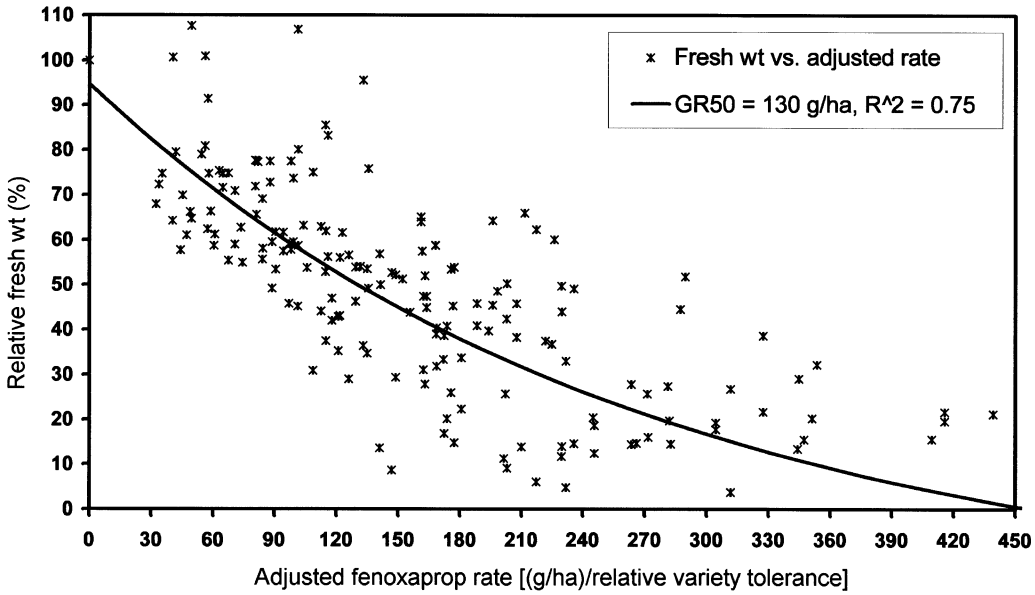

FIGURE 1. Italian ryegrass (*Lolium multiflorum* Lam.) response to fenoxaprop applied at the three-leaf growth stage. The fenoxaprop rate was adjusted by dividing the applied dose by the relative tolerance of each individual cultivar from Table 1. Relative fresh wt = $-20.45 + 115.2 \times 0.9962^{(\text{adjusted rate})}$, with $R^2 = 0.75$ and $GR_{50} = 130$ g ha^{-1} for a cultivar with a relative tolerance of 1. The GR_{50} is correspondingly higher or lower if the cultivar relative tolerance is greater than or less than 1, respectively.

TABLE 2. Effect of fenoxaprop applied at the two-leaf and four-leaf growth stages to 10 Italian ryegrass (*Lolium multiflorum* Lam.) cultivars grouped by tolerance.[a]

Cultivar tolerance group (*n*) and growth stage[b]	Mean relative fresh wt[c]	GR_{50}	R^2	Regression MSE	*F* test[d]	Relative tolerance[e]
	% of check	g ha^{-1}				
Sensitive (4) at two-leaf	33 c	108	0.49	812	60**	0.5
Sensitive (4) at four-leaf	37 c	134	0.48	941	57**	0.6
Intermediate (2) at two-leaf	48 b	177	0.58	710	35**	0.8
Intermediate (2) at four-leaf	56 b	212	0.47	579	23**	1.0
Tolerant (4) at two-leaf	56 b	215	0.37	687	44**	1.0
Tolerant (4) at four-leaf	75 a	450	0.22	633	22**	2.1
Averages within cultivar tolerance groups (*n*)						
Sensitive (4)	35 c	121				0.6
Intermediate (2)	52 b	195				0.9
Tolerant (4)	66 a	332				1.5
Regression of data pooled within cultivar tolerance groups (*n*)						
Sensitive (4)		122	0.48	870	115**	0.6
Intermediate (2)		192	0.52	641	58**	0.9
Tolerant (4)		321	0.27	719	61**	1.5
Averages within leaf stage groups						
Two-leaf stage	46 b	167				0.8
Four-leaf stage	56 a	265				1.2
Regression of data pooled within leaf stage groups						
Two-leaf stage		160	0.39	838	99**	0.7
Four-leaf stage		254	0.24	968	55**	1.2
Regression of data adjusted as fenoxaprop rate divided by relative leaf stage and cultivar tolerance						
All cultivars (10), both stages		211	0.47	705	198**	1.0

[a] Abbreviations: MSE, mean square error; wt, weight.

[b] Sensitive group includes Futaharu, Ace, Waseyutaka, and Tetrone; intermediate group includes Sakurawase and Barmultra; tolerant group includes Gulf, Aubade, Marshall, and Torero. Number of cultivars within each group is denoted by *n*.

[c] Mean relative fresh wt is the average of the data from all rates of fenoxaprop that averaged 168 g ha^{-1}. Means followed by the same letter do not differ at the P = 0.05 probability level.

[d] Regressions based on pooled data from five to seven rates of fenoxaprop in two experiments. The symbols * and ** denote regression *F* test significance at the P = 0.05 and 0.01 levels, respectively.

[e] Relative tolerance is calculated as GR_{50} of the cultivar or group of cultivars divided by mean GR_{50}.

TABLE 3. Effect of fenoxaprop applied at the two-leaf, four-leaf, and tillered growth stages to five Italian ryegrass (*Lolium multiflorum* Lam.) cultivars.[a]

Cultivar and growth stage	Mean relative fresh wt[b]	GR_{50}	R^2	Regression MSE	*F* test[c]	Relative tolerance[d]
	% of check	g ha^{-1}				
Futaharu at two-leaf	31 ef	50	0.92	99	31**	0.3
Futaharu at four-leaf	13 f	52	0.94	158	54**	0.3
Futaharu tillered	65 abc	172	0.83	334	24**	0.9
Ace at two-leaf	48 cde	103	0.68	856	8*	0.5
Ace at four-leaf	12 f	40	0.96	74	179**	0.2
Ace tillered	56 bcd	179	0.52	345	5 +	1.0
Aubade at two-leaf	57 bcd	154	0.92	54	12*	0.8
Aubade at four-leaf	69 ab	256	0.34	248	2 NS	1.3
Aubade tillered	59 bcd	193	0.37	747	2 NS	1.0
Gulf at two-leaf	71 ab	246	0.89	99	76**	1.3
Gulf at four-leaf	83 a	389	0.85	94	27**	2.0
Gulf tillered	86 a	489	0.62	170	8*	2.5
Marshall at two-leaf	53 bcd	169	0.71	251	8*	0.9
Marshall at four-leaf	43 de	180	0.17	610	1 NS	0.9
Marshall tillered	73 ab	266	0.75	293	23**	1.4
Averages within growth stage groups						
Two-leaf stage	52 b	144				0.7
Four-leaf stage	44 b	183				0.9
Tillered	68 a	260				1.3

[a] Abbreviations: MSE, mean square error; wt, weight; NS, not significant.

[b] Mean relative fresh wt is the average of the data from all rates of fenoxaprop that averaged 146 g ha^{-1}. Means followed by the same letter do not differ at the $P = 0.05$ probability level.

[c] Regressions based on data from six rates of fenoxaprop in a single experiment. The symbols +, *, and ** denote regression *F* test significance at the P = 0.10, 0.05 and 0.01 levels, respectively.

[d] Relative tolerance is calculated as GR_{50} of the cultivar or at the indicated growth stage divided by mean GR_{50}.

among the cultivars and the shape of the response curve both suggest the presence of multiple genetic factors controlling the tolerance to fenoxaprop. In contrast, two or three discrete categories of response would be expected if a single gene, such as that coding for an altered enzyme, conditioned the level of tolerance.

Growth Stage Effects

Italian ryegrass was more sensitive to fenoxaprop at the two-leaf stage than at the four-leaf growth stage. The GR_{50} values increased by 23, 20, and 110% in the sensitive, intermediate, and tolerant groups, respectively, between the two-leaf and four-leaf growth stages (Table 2). The GR_{50} value was 59% higher at the four-leaf stage than at the two-leaf stage both for averages over regressions within the growth stage and the tolerance group and for regressions of data pooled over tolerance groups. The 10 Italian ryegrass cultivars used in the growth stage studies showed slightly better tolerance to fenoxaprop than they had shown in the earlier studies of all 21 cultivars. Reasons for improved tolerance are unclear but probably relate to the differences in growing conditions between tests. One of the three growth stage tests was conducted during the winter, whereas the other two were conducted during summer, unlike the original cultivar tests that were both run in spring. Variances were also larger in the growth stage tests than in the original cultivar tests. Increased variation may have been caused by sporadic temperature and moisture stress during summer, differences in light intensity between tests, and longer periods of time between planting and herbicide application needed to achieve desired growth stages during winter. The increase in tolerance with growth stage suggested that the extent of injury by fenoxaprop and ultimate recovery or death was controlled by metabolic factors, such as concentration and activity of the enzymes inhibited by this herbicide and the enzymes involved in detoxification of fenoxaprop. Studies conducted by other researchers subsequent to our own work have identified two isoforms of ACCase in Italian ryegrass with differing sensitivity to APP and CHD herbicides and differing contribution to total ACCase activity depending on the growth stage (Evenson et al. 1997).

The tillered growth stage was included in the final growth stage by cultivar test to explore whether Italian ryegrass continued to increase in tolerance beyond that present at the four-leaf growth stage. Averaged over all five cultivars, the GR_{50} value at the tillered stage was 42% higher than that at the four-leaf stage, and 80% higher than that at the two-leaf stage (Table 3). The response of Marshall at the four-leaf growth stage in this test was erratic and inconsistent with the results from the earlier tests. The nonsignificant regression *F* test for Marshall at the four-leaf growth stage may indicate possible treatment misapplication, data recording error, or genotype by environment interaction. The cultivar Aubade displayed unusual response by demonstrating a level of tolerance to fenoxaprop that did not increase with growth stage. Indeed, regressions at the four-leaf and tillered stages for Aubade were nonsignificant, implying that most of the fenoxaprop rates used were too low to cause severe injury. Aubade was more sensitive than Gulf at the tillered growth stage. Another possible explanation for the response of Aubade is that this cultivar might contain a segregating mixture of a small proportion of susceptible genes within a

TABLE 4. Acetyl–coenzyme A carboxylase (ACCase) specific activity and I_{50} for inhibition by fenoxaprop for 11 Italian ryegrass (*Lolium multiflorum* Lam.) cultivars.[a]

Cultivar	Specific activity of ACCase[b]	I_{50} for the inhibition of ACCase by fenoxaprop[c]	Relative tolerance[d]
	nmol ^{14}C-acetate mg protein^{-1} min^{-1}	μm	
Futaharu	5.5 bc	0.20 ab	0.5
Ace	9.0 a	0.09 b	0.5
Waseyutaka	8.2 ab	0.17 ab	0.6
Tetrone	8.3 ab	0.15 b	0.6
Billiken	8.8 a	0.11 b	0.7
Sakurawase	4.8 c	0.19 ab	1.0
Barmultra	7.6 abc	0.15 b	1.0
Gulf	7.4 abc	0.20 ab	1.3
Aubade	8.4 ab	0.11 b	1.3
Ellire	5.7 bc	0.19 ab	1.4
Marshall	6.7 abc	0.26 a	1.7

[a] Means followed by the same letter within a column do not differ at the P = 0.05 probability level.
[b] Mean of six determinations for two youngest leaves harvested at the tillered growth stage.
[c] Mean of 10 (Ace, Tetrone, Billiken, Sakurawase, Barmultra, and Ellire) or 22 (Futaharu, Waseyutaka, Aubade, Gulf, and Marshall) determinations.
[d] Relative tolerance obtained by testing 21 cultivars (Table 1).

generally tolerant germplasm. Because Italian ryegrass cultivars are open-pollinated populations, all cultivars potentially contain genes segregating for traits, such as tolerance to fenoxaprop. As a weed in cereal crops, Italian ryegrass rapidly developed resistance to diclofop despite initial susceptibility to this ACCase inhibitor (Stanger and Appleby 1989).

Acetyl–Coenzyme A Carboxylase

The specific activity of ACCase extracted at the tillered stage from plants not treated with fenoxaprop differed among the 11 cultivars tested (Table 4). Higher activity was found in the susceptible cultivars Ace, Waseyutaka, Tetrone, and 'Billiken', in the intermediate cultivar Barmultra, and in the tolerant cultivars Gulf, Aubade, and Marshall. Lower activity was found in the susceptible cultivar Futaharu, the intermediate cultivar Sakurawase, and the tolerant cultivar Ellire. Activity varied by slightly less than twofold between the extremes, Ace and Sakurawase. Although this pattern suggested no clear relationship between the specific activity of ACCase and the whole-plant tolerance to fenoxaprop, magnitudes of the differences were comparable.

The I_{50} value for the inhibition of the extracted ACCase by fenoxaprop varied among the cultivars by a factor of nearly threefold (Table 4). The enzyme found in Marshall was more tolerant to fenoxaprop than the form found in Ace, Tetrone, Billiken, Barmultra, and Aubade. The I_{50} values for the inhibition of the ACCase extracted from Futaharu, Waseyutaka, Sakurawase, Gulf, and Ellire were numerically intermediate and did not differ statistically from the I_{50} values for any of the cultivars. As in the case of specific activity, there was no clear relationship between the I_{50} value and the whole-plant tolerance. However, Marshall did have the highest I_{50} value along with an intermediate level of specific activity, whereas most of the sensitive cultivars were either low in specific activity or had a low I_{50} value. Another possibility is that the plants may possess more than just a single version of the ACCase. In such a case, differential inhibition or activity (or both) of the ACCase pools must be added to differential induction as potential causes for variation in whole-plant tolerance to fenoxaprop.

The specific activity of ACCase at the tillered stage averaged threefold greater than that at the two- and four-leaf stages, which did not differ from each other (Table 5). This increased activity is somewhat greater than the difference in relative tolerance between tillered and younger growth stages, and it suggests a probable role for the specific activity of ACCase in growth stage–related tolerance. There was no run by treatment (growth stage or cultivar) interaction for the ACCase activity when expressed per unit protein, although there were significant main effects of runs and run by treatment interactions when the activity was expressed per unit tissue fresh wt (data not shown). There was no measurable

TABLE 5. Mean acetyl–coenzyme A carboxylase (ACCase) specific activity and I_{50} for inhibition by fenoxaprop for five Italian ryegrass (*Lolium multiflorum* Lam.) cultivars at three growth stages.[a]

Growth stage	Specific activity of ACCase[b]	I_{50} for the inhibition of ACCase by fenoxaprop[b]	Relative tolerance[c]
	nmol ^{14}C-acetate mg protein^{-1} min^{-1}	μm	
Two-leaf	1.7 b	0.2 a	0.7
Four-leaf	1.6 b	0.25 a	0.9
Tillered	5.1 a	0.19 a	1.3

[a] Means followed by the same letter within a column do not differ at the P = 0.05 probability level.
[b] Mean of 20 determinations (four per cultivar).
[c] Relative tolerance obtained by testing the cultivars at the two-leaf, four-leaf, and tillered growth stages (Table 3).

change in the I_{50} value between growth stages. It would be useful to measure the specific activity of ACCase in extracts from treated leaves to determine whether tolerant and sensitive cultivars differ in their ability to maintain the function of this critical enzyme during the first few weeks after application.

Because the GR_{50} value at the three-leaf growth stage for even the most tolerant cultivar was only 228 g ha^{-1}, the fenoxaprop label was amended to reduce the maximum rate of application on Italian ryegrass to 168 g ha^{-1} and to limit its use on the more tolerant cultivars. The manufacturer recently decided to stop the production of several formulations of fenoxaprop, including the only one registered for use on cool-season grasses grown for seed. Questions of crop safety and cultivar variation in tolerance will need to be reexamined for new formulations of fenoxaprop enriched in the active isomer and containing chemical safeners. Specific Italian ryegrass cultivars will have to be chosen for new tests of fenoxaprop safety. Oregon common remains the most widely grown type of Italian ryegrass and would clearly merit testing. Of the 21 cultivars included in the tests being reported in this article, eight remained in production during the most recent 3 yr, six others were still on the list of those eligible for certification, whereas the final seven have been dropped from both production and certification eligibility. A total of 40 Italian ryegrass cultivars were eligible for certification in 2001, 13 of which were included in the original selection of the 21 cultivars.

Sources of Materials

[1] New England Nuclear-Dupont/PerkinElmer Life Sciences Inc., 549 Albany Street, Boston, MA 02118-2512.

[2] ICN, Biomedicals Inc., 2727 Campus Drive, Irvine, CA 92612.

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Literature Cited

Andersen, R. N. 1976. Response of monocotyledons to HOE 22870 and HOE 23408. Weed Sci. 24:266–269.

Betts, K. J., N. J. Elke, D. L. Wyse, J. W. Gronwald, and D. A. Somers. 1992. Mechanism of inheritance of diclofop resistance in Italian ryegrass (*Lolium multiflorum*). Weed Sci. 40:184–189.

Bhowmik, P. C. 1986. Fenoxaprop-ethyl for post emergence control in Kentucky bluegrass turf. Hortscience 21:457–458.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72:248–254.

Brewster, B. D., A. P. Appleby, and R. L. Spinny. 1977. Control of Italian ryegrass and wild oats in winter wheat with HOE 23408. Agron. J. 69:911–913.

Butler, J.H.B. and A. P. Appleby. 1986. Tolerance of red fescue (*Festuca rubra*) and bentgrass (*Agrostis* spp.) to sethoxydim. Weed Sci. 34:457–461.

De Prado, J. L., R. A. De Prado, and R. H. Shimabukuro. 1999. The effect of diclofop on membrane potential, ethylene induction, and herbicide phytotoxicity in resistant and susceptible biotypes of grasses. Pestic. Biochem. Physiol. 63:1–14.

De Prado, R., J. González-Gutiérrez, J. Menéndez, J. Gasquez, J. W. Gronwald, and R. Giménez-Espinosa. 2000. Resistance to acetyl CoA carboxylase–inhibiting herbicides in *Lolium multiflorum*. Weed Sci. 48: 311–318.

Evenson, K. J., J. W. Gronwald, and D. L. Wyse. 1994. Purification and characterization of acetyl-coenzyme A carboxylase from diclofop-resistant and -susceptible *Lolium multiflorum*. Plant Physiol. 105:671–680.

Evenson, K. J., J. W. Gronwald, and D. L. Wyse. 1997. Isoforms of acetyl-coenzyme A carboxylase in *Lolium multiflorum*. Plant Physiol. Biochem. 35:265–272.

Gronwald, J. W. 1991. Lipid biosynthesis inhibitors. Weed Sci. 39:435–449.

Gronwald, J. W., C. V. Eberlein, K. J. Betts, R. J. Baerg, N. J. Elke, and D. L. Wyse. 1992. Mechanism of diclofop resistance in an Italian ryegrass (*Lolium multiflorum* Lam.) biotype. Pestic. Biochem. Physiol. 44:126–139.

Harwood, J. L. 1989. The properties and importance of acetyl-coenzyme A carboxylase in plants. Proc. Br. Crop Prot. Conf.—Weeds 1:155–162.

Köcher, H., H. M. Kelner, K. Lötzch, E. Dorn, and O. Wink. 1982. Mode of action and metabolic fate of the herbicide fenoxaprop-ethyl, HOE33171. Proc. Br. Crop Prot. Conf.—Weeds 1:341–347.

Kuk, Y., N. R. Burgos, and R. E. Talbert. 2000. Cross- and multiple resistance of diclofop-resistant *Lolium* spp. Weed Sci. 48:412–419.

Matthews, J. M., J.A.M. Holtum, D. R. Liljegren, B. Furness, and S. B. Powles. 1990. Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). I. Properties of the herbicide target enzymes acetyl-coenzyme A carboxylase and acetolactate synthase. Plant Physiol. 94: 1180–1186.

Menéndez, J., J. L. De Prado, and R. De Prado. 1996. Diclofop-methyl metabolism in resistant biotypes of *Lolium rigidum*. Pages 97–98 *in* R. De Prado, J. Jorrín, L. García-Torres, and G. Marshall, eds. Proceedings of the International Symposium on Weed and Crop Resistance to Herbicides. Córdoba, Spain: University of Córdoba.

Morrison, I. A. and D. C. Maurice. 1984. The relative response of two foxtail (*Setaria*) species to diclofop. Weed Sci. 32:686–690.

Mueller-Warrant, G. W. 1990. Control of roughstalk bluegrass (*Poa trivialis*) with fenoxaprop in perennial ryegrass (*Lolium perenne*). Weed Technol. 4:250–257.

Mueller-Warrant, G. W. 1991. Enhanced activity of single-isomer on several cool-season grasses. Weed Technol. 5:826–833.

Palmer, J. J. and M. A. Read. 1991. Fenoxaprop-ethyl: a summary of UK trials on grass weed control in wheat. Proc. Br. Crop Prot. Conf.—Weeds 3:945–952.

Parker, W. P., D. A. Somers, D. L. Wyse, R. A. Keith, J. D. Burton, J. W. Gronwald, and B. G. Gengenbach. 1990. Selection and characterization of sethoxydim-tolerant maize tissue cultures. Plant Physiol. 92: 1220–1225.

Powles, S. B., J.A.M. Holtum, J. M. Matthews, and D. R. Liljegren. 1990. Herbicide cross-resistance in annual ryegrass (*Lolium rigidum* Gaud): the search for a mechanism. Pages 394–406 *in* M. B. Green, H. M. Lebaron, and W. K. Moberg, eds. Managing Resistance to Agrochemicals: From Fundamental Research to Practical Strategies. ACS Symposium Series No. 421. Washington, DC: American Chemical Society.

Preston, C., F. J. Tardif, J. T. Christopher, and S. B. Powles. 1996. Multiple resistance to dissimilar herbicide chemistries in a biotype of *Lolium rigidum* due to enhanced activity of several herbicide degrading enzymes. Pestic. Biochem. Physiol. 54:123–134.

Rendina, A. R., J. D. Beaudoin, A. C. Kraig-Kennard, and M. K. Breen. 1989. Kinetics of inhibition of acetyl-coenzyme A by the aryloxyphenoxypropionate and cyclohexanedione graminicides. Proc. Br. Crop Prot. Conf.—Weeds 1:163–172.

Rendina, A. R. and J. M. Felts. 1988. Cyclohexanedione herbicides are selective and potent inhibitors of acetyl-CoA carboxylase from grasses. Plant Physiol. 86:983–986.

Schumacher, H., M. Rottele, and R. J. Marrese. 1982. Grass weed control in soybeans with HOE 33171. Proc. Br. Crop Prot. Conf.—Weeds 2: 703–708.

Secor, J. and C. Csèke. 1988. Inhibition of acetyl-CoA carboxylase activity by haloxyfop and tralkoxydim. Plant Physiol. 86:10–12.

Shah, D. M., R. B. Horsch, H. K. Klee, et al. 1986. Engineering herbicide tolerance in transgenic plants. Science 233:478–481.

Shimabukuro, R. H. 1990. Selectivity and mode of action of the post-

emergence herbicide diclofop-methyl. Plant Growth Regulator Soc. Am. Quart. 18:37–54.
Shimabukuro, R. H. and B. L. Hoffer. 1991. Metabolism of diclofop-methyl in susceptible and resistant biotypes of *Lolium rigidum*. Pestic. Biochem. Physiol. 39:251–260.
Shimabukuro, R. H., W. C. Walsh, and R. A. Hoerauf. 1979. Metabolism and selectivity of diclofop-methyl in wild oat and wheat. J. Agric. Food Chem. 27:615–623.
Stahl, R. J. and S. A. Sparace. 1991. Characterization of fatty acid biosynthesis in isolated pea root plastids. Plant Physiol. 96:602–608.
Stanger, C. E. and A. P. Appleby. 1989. Italian ryegrass (*Lolium multiflorum*) accessions tolerant to diclofop. Weed Sci. 37:350–352.
Stoltenberg, D. E., J. W. Gronwald, D. L. Wyse, J. D. Burton, D. A. Somers, and B. G. Gengenbach. 1989. Effect of sethoxydim and haloxyfop on acetyl-coenzyme A carboxylase activity in *Festuca* species. Weed Sci. 37:512–516.

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